Cell

Distinct Ubiquitin-Ligase Complexes Define Convergent Pathways for the Degradation of ER Proteins

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DOI 10.1016/j.cell.2006.05.043

SUMMARY

Many misfolded endoplasmic reticulum (ER) proteins are eliminated by ERAD, a process in which substrates are polyubiquitylated and moved into the cytosol for proteasomal degradation. We have identified in *S. cerevisiae* distinct ubiquitin-ligase complexes that define different ERAD pathways. Proteins with misfolded ER-luminal domains use the ERAD-L pathway, in which the Hrd1p/Hrd3p ligase forms a near stoichiometric membrane core complex by binding to Der1p via the linker protein Usa1p. This core complex associates through Hrd3p with Yos9p, a substrate recognition protein in the ER lumen. Substrates with misfolded intramembrane domains define a pathway (ERAD-M) that differs from ERAD-L by being independent of Usa1p and Der1p. Membrane proteins with misfolded cytosolic domains use the ERAD-C pathway and are directly targeted to the Doa10p ubiquitin ligase. All three pathways converge at the Cdc48p ATPase complex. These results lead to a unifying concept for ERAD that may also apply to mammalian cells.

INTRODUCTION

Misfolded proteins accumulating in the lumen or membrane of the endoplasmic reticulum (ER) cause the unfolded protein response (UPR), a collection of signaling pathways that adapt cells to ER stress (Travers et al., 2000). If the problem cannot be rectified, these proteins are eventually transported into the cytosol and degraded by the proteasome. This process is called ERAD (for ER-associated protein degradation), retrotranslocation, or dislocation (Meusser et al., 2005; Tsai et al., 2002), and it plays a role in many diseases. The mechanism of ERAD is still largely unknown, but it involves several different steps (for review, see Meusser et al., 2005; Tsai et al., 2002). The process begins with the recognition of a substrate as being misfolded, likely involving certain chaperones. Next, the substrate is transported across the ER membrane, a process that, at least for luminal proteins, probably requires a protein-conducting channel. Once on the cytoplasmic side of the membrane, most substrates are polyubiquitylated in a process that requires specific ubiquitin-conjugating and -ligase enzymes. The polyubiquitin chain is subsequently recognized by a cytosolic ATPase complex, consisting of an ATPase, called p97 or VCP in mammals and Cdc48p in yeast, and a heterodimeric cofactor, containing Ufd1p and Npl4p. The hydrolysis of ATP is required to move the polypeptide into the cytosol so that it can be degraded by the proteasome.

Although most ERAD substrates follow this general scheme, they appear to use distinct pathways, which are best characterized in *S. cerevisiae* (Huyer et al., 2004; Vashist and Ng, 2004). Proteins with misfolded cytosolic domains (ERAD-C substrates) are degraded rapidly and require the ubiquitin ligase Doa10p, a multispanning membrane protein with a cytosolic RING finger domain (Swanson et al., 2001). Proteins with misfolded luminal domains (ERAD-L substrates) are degraded more slowly and might have to cycle through the *cis*-Golgi compartment before undergoing retrotranslocation at the ER (Caldwell et al., 2001; Vashist et al., 2001). The recognition of these substrates involves luminal chaperones, including the lectin-like proteins Yos9p and Htm1p (Bhamidipati et al., 2005; Buschhorn et al., 2004; Jakob et al., 2001; Kim et al., 2005; Szathmary et al., 2005). The ERAD-L pathway requires the ubiquitin ligase Hrd1p (Der3p) for substrate polyubiquitylation (Bays et al., 2001; Bordallo et al., 1998; Hampton et al., 1996). Hrd1p is also a multispanning membrane protein with a cytosolic RING finger domain; it is associated with Hrd3p, a membrane protein of unknown function with a sizable luminal domain (Gardner et al., 2000). Both ERAD-C and -L pathways employ the Cdc48p/Ufd1p/Npl4p ATPase complex and the recently identified adaptor protein Ubx2p, a membrane protein that binds Cdc48p through its cytosolic Ubx domain (Neuber et al., 2005; Schuberth and Buchberger, 2005).

The precise mechanistic differences between the ERAD-C and ERAD-L pathways are unknown. The

number of identified components required for the degradation of various substrates is bewildering and no coherent picture has yet emerged. In addition, the precise function of many components is unknown and that of others is controversial. A particularly important issue is the nature of the protein-conducting channel, which likely is required for at least soluble ERAD-L substrates. One possibility is that retrotranslocation occurs through the same Sec61p channel that is used for the transport of proteins in the "forward" direction, from the cytosol into the ER. However, the experiments implicating Sec61p in ERAD are not entirely convincing (for a discussion, see Tsai et al., 2002), and other candidates have been proposed, including the ubiquitin ligase Hrd1p (Gauss et al., 2006) and Der1p (Lilley and Ploegh, 2004; Ye et al., 2004), a membrane protein that spans the membrane four times (Knop et al., 1996). In summary, although it is now clear that ERAD substrates employ different routes, the identification of the participating components and their arrangements into pathways remain important issues. In addition, it is unclear whether proteins with misfolded intramembrane domains constitute yet another class of ERAD substrates, and if so, which components are involved.

The situation in mammalian cells is even less clear, although many ERAD components are sequence-related to yeast proteins. Perhaps the best characterized pathway is the one in which ERAD is hijacked by the US11 protein of the human cytomegalovirus to destroy newly synthesized MHC class I heavy chains, allowing the virus to evade the cellular immune defense. The single-spanning membrane protein US11 first interacts with the MHC class I heavy chain in the ER lumen. US11 then interacts with Derlin-1, a protein with sequence similarity to the yeast Der1p (Lilley and Ploegh, 2004; Ye et al., 2004). Derlin-1 associates with the ubiquitin ligases Hrd1p and gp78 (Lilley and Ploegh, 2005; Ye et al., 2005), which are both related to yeast Hrd1p, but these ligases do not seem to be required for the degradation of MHC class I molecules (Kikkert et al., 2004). The cytosolic p97 ATPase and its cofactor Ufd1p/Npl4p are recruited by interactions with several different membrane proteins, which include Derlin-1, the ligases Hrd1p and gp78, and VIMP, a Derlin-1-associated protein that lacks obvious homologs in lower eukaryotes (Ye et al., 2004, 2005). Surprisingly, a related pathway, in which the viral protein US2 triggers the degradation of MHC class I heavy chains, does not seem to employ Derlin-1 (Lilley and Ploegh, 2004). While cellular substrates for Derlin-1 have yet to be identified, one ERAD substrate has been identified that requires the related proteins Derlin-2 and -3 (Oda et al., 2006). Additional components may also play a role, although their functions are even less clear. These include Herp, a membrane protein that is highly upregulated by the UPR, binds to Hrd1p, and is required for the degradation of some ERAD substrates (Kokame et al., 2000; Schulze et al., 2005). The existence of multiple genes for many mammalian components, the difficulty to make knockouts in mammalian cells, and the lack of substrates are among the factors that make it difficult to derive a general concept for mammalian ERAD.

Here we have taken a systematic approach in *S. cerevisiae* to identify several different ubiquitin-ligase complexes, which define distinct pathways for the degradation of ER proteins with misfolded domains in the lumen, the membrane, or the cytosol. The proposed unifying concept of ERAD may also apply to mammalian cells.

RESULTS

Interaction Partners of the Two ERAD Ubiquitin Ligases

We performed a systematic analysis of the interactions of the two major ubiquitin ligases implicated in ERAD in *S. cerevisiae*. The ligase Doa10p was C-terminally fused to a tandem affinity purification (TAP) tag, consisting of a calmodulin binding peptide and an IgG interaction module, separated by a TEV cleavage site (Rigaut et al., 1999). The tagged protein was expressed from its endogenous promoter and was functional, as assayed by the degradation of the standard ERAD-C substrate Ste6-166p (Huyer et al., 2004; Figure S1). The Doa10p-TAP was isolated from a crude membrane fraction by solubilization in the detergent digitonin, followed by affinity purification. Initially, we employed the usual two-step procedure using IgG and calmodulin columns, but we noticed that higher yields could be obtained by single-step purification on IgG-coupled magnetic beads without compromising the purity of the samples. The eluted proteins were subjected to SDS-PAGE and showed several bands that were not seen in mock-purified samples from wild-type cells (Figure 1A). Tandem mass spectroscopy identified the Doa10p-interacting proteins as the ubiquitin-conjugating enzyme Ubc7p and its membrane anchor Cue1p (Biederer et al., 1997), the ATPase Cdc48p, its cofactor Npl4p, and Ubx2p, a membrane-recruiting factor for Cdc48p. All these components have been implicated in the degradation of ERAD-C substrates. Similar results were obtained by mass spectroscopy of the total population of TAP-purified proteins, analyzed without separation by SDS-PAGE (Figures 1B and S2). The composition of the Doa10p complex was confirmed by TAP purification of C-terminally tagged Ubx2p (Figure 1B), again expressed from its endogenous promoter and fully functional in degrading a known ERAD substrate (Figure S1). These experiments suggest that the Doa10p complex is relatively simple, consisting of ubiquitin-conjugating and -ligating enzymes and the Cdc48p ATPase complex, including its membrane-recruiting factor Ubx2p.

A similar strategy was followed to identify interaction partners of the ubiquitin ligase Hrd1p. Hrd1p-TAP was expressed from its endogenous promoter and was fully functional in degrading the misfolded luminal protein CPY*, an ERAD-L substrate (Vashist and Ng, 2004; Figure S1). Several Hrd1p-interacting proteins were identified by SDS-PAGE and mass spectrometry (Figure 2A). As expected, we found the Hrd1p cofactor Hrd3p (Gardner et al., 2000),

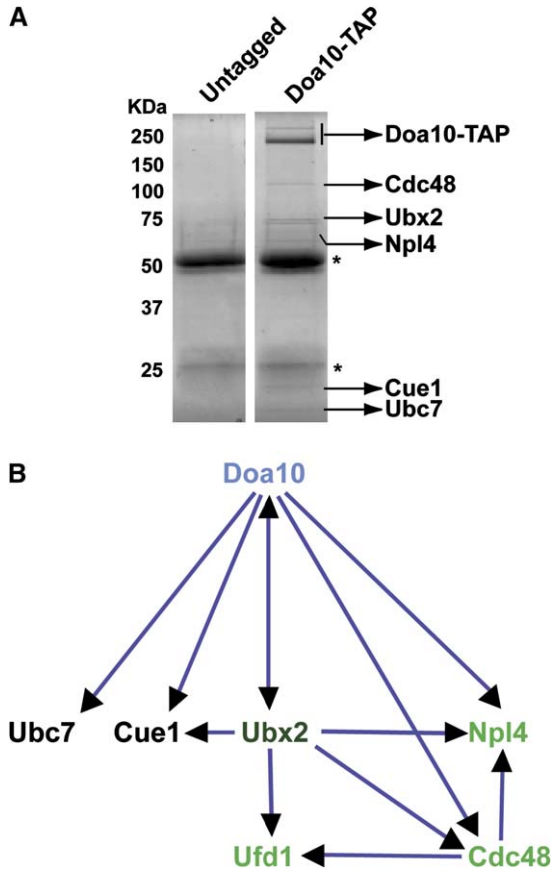

Figure 1. Ubiquitin Ligase Doa10p-Associated Proteins

(A) TAP-tagged Doa10p (Doa10-TAP) was purified from digitonin-solubilized membranes using IgG-coupled magnetic beads. SDS-eluted proteins were separated by SDS-PAGE and stained with Coomassie blue. A control was performed with wild-type cells ("untagged"). The identity of the proteins in the bands was determined by mass spectrometry (Figure S2). Stars indicate the positions of IgG heavy and light chains.

(B) The network represents a summary of all identified interactions, using Doa10-TAP, Ubx2-TAP, or Cdc48-TAP as baits. The interaction partners were pulled down with IgG-coupled magnetic beads and identified by mass spectrometry without further separation. In the case of Doa10-TAP, partners were also identified from bands in SDS-PAGE (see Figures 1A and S2). Arrows point from the bait to the interaction partner. Components in black and light green indicate ubiquitin-conjugating and Cdc48p-ATPase complexes, respectively.

Ubx2p and Cdc48p (Neuber et al., 2005; Schuberth and Buchberger, 2005), and Der1p (Gauss et al., 2006). In addition, we identified Yos9p, a lectin-like luminal protein proposed to deliver substrates to the ERAD machinery (Bhamidipati et al., 2005; Kim et al., 2005; Szathmary et al., 2005), and a protein called Usa1p. Usa1p is a membrane protein of ~100 kDa of hitherto unknown function (Awasthi et al., 2001). It is encoded by a nonessential gene and will be discussed in more detail in a later section. The same binding partners of Hrd1p-TAP were identified when the proteins eluted from the IgG column were analyzed without further separation (Figure S2). To confirm the interactions, we purified TAP fusions of Hrd3p, Yos9p, Der1p, Usa1p, Ubx2p, and Cdc48p. The tag compromised the function of these proteins to varying degrees, as assayed by the degradation of CPY* (Figure S1); the Hrd3p and Der1p fusions were practically nonfunctional. Nevertheless, all tagged proteins were still capable of interacting with the other components of the complex (Figure 2B). When the Usa1p-interacting proteins were separated by SDS-PAGE, all major bands corresponded to the proteins of the complex (Figure 2C). Usa1p and Hrd3p have nearly identical sizes, but the TAP-tag on Usa1p allowed their separation (compare Figures 2A and 2C). From the relative intensities of the Coomassie-stained bands it appears that Hrd1p, Hrd3p, and Usa1p are present in near stoichiometric amounts.

For both the Doa10p and Hrd1p complexes, all identified components were represented by a large set of peptides, which often covered much of the entire protein sequence (Figure S2). The only exception was Der1p, for which only a few tryptic peptides were found, in agreement with the predictions from its sequence. Most purifications were performed at least twice and gave the same major interaction partners (Figure S2). Contaminating proteins, on the other hand, were variable and generally gave only a few peptides. We therefore feel confident that we have identified most, if not all, components of the two ubiquitin-ligase complexes. The identification of the proteins in both Coomassie-stained SDS gels and in unfractionated samples also argues that the interactions among the components are direct rather than mediated by some unidentified component.

Characterization of the Hrd1p Complex

Given the large number of components interacting with Hrd1p, we wished to test whether they are all present in a high-molecular-weight complex. To this end, we used strains expressing either tagged or endogenous proteins, isolated membrane fractions, and subjected digitonin extracts prepared from them to sucrose gradient centrifugation. Individual fractions were analyzed by immunoblotting. We found that Hrd1p-TAP migrated at a position corresponding to a molecular weight of >500 MDa (Figures 3A and S3), much larger than expected from its actual size. Endogenous Hrd1p sedimented at the same position, as determined with Hrd1p antibodies (data not shown). When SDS was added prior to sucrose gradient centrifugation, Hrd1-TAP shifted to a much smaller size (Figure 3B), supporting the idea that Hrd1p is present in a complex. Hrd3p-13Myc showed a similar profile to Hrd1p (Figure 3C). In parallel experiments we found that Der1p-TAP fractionated into two peaks: one matching that of Hrd1p and the other corresponding to a significantly smaller size (Figure 3D). The same two populations were seen with Usa1p-TAP (Figure 3E) or with endogenous Usa1p (Figure 3F), analyzed by immunoblotting with antibodies raised against the protein. In contrast, Yos9p-13Myc, which was functional (Figure S1), showed a broad

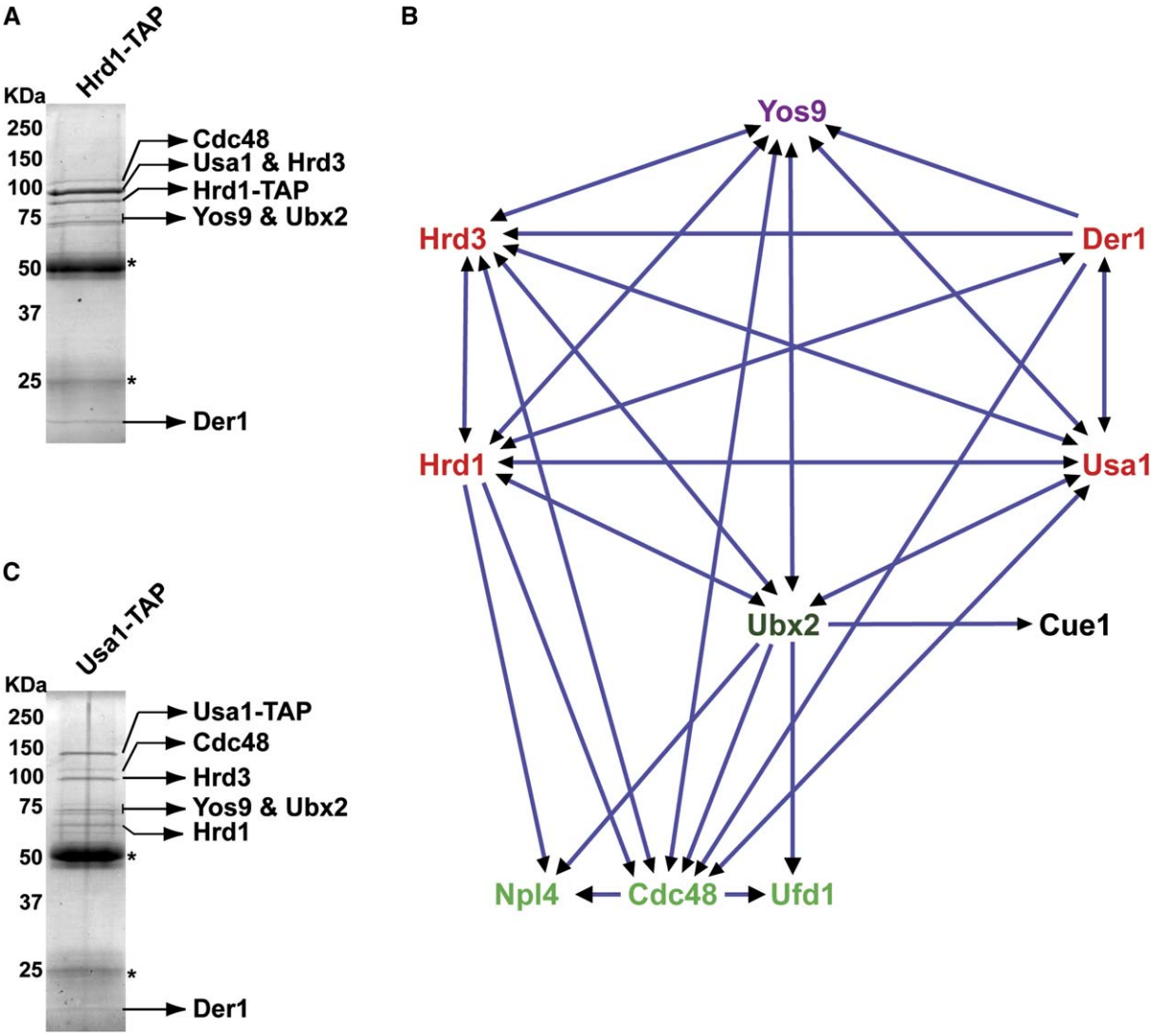

Figure 2. Ubiquitin Ligase Hrd1p-Associated Proteins

(A) TAP-tagged Hrd1p (Hrd1-TAP) was purified from digitonin-solubilized membranes using IgG-coupled magnetic beads. SDS-eluted proteins were separated by SDS-PAGE and stained with Coomassie blue. The bands indicated were not seen in a mock-purification and were identified by mass spectrometry. Stars (*) indicate the positions of IgG heavy and light chains.

(B) The network represents a summary of all identified interactions, using Hrd1-TAP, Hrd3-TAP, Yos9-TAP, Der1-TAP, Usa1-TAP, Ubx2-TAP, or Cdc48-TAP as baits. The interaction partners were pulled down with IgG-coupled magnetic beads and identified by mass spectrometry without further separation. In the case of Hrd1-TAP and Usa1-TAP, partners were also identified from bands in SDS-PAGE (see Figures 2A, 2C and S2). Arrows point from the bait to the interaction partner. Components in red and light green are in the Hrd1p-core and Cdc48p-ATPase complexes, respectively.

(C) As in (A), but with Usa1p-TAP as a bait.

distribution, with only a small population having a large size (Figure 3G). Ubx2p-TAP (Figure 3H) or endogenous Cdc48p (Figure 3I) sedimented almost exclusively at lower molecular weight fractions, although they did not exactly comigrate. Components of the 19S regulatory particle of the proteasome were also found in these fractions (data not shown). Taken together, these results suggest that Hrd1p, Hrd3p, Usa1p, and Der1p are present in a large complex, which we will refer to as the Hrd1p core complex. Mammalian Hrd1p is also in a large complex (Schulze et al., 2005). The other proteins detected in the pull-down experiments (Yos9p, Ubx2p, and Cdc48p) could either be present in excess of the core components or have a lower affinity to them. The size of the core complex appears to be larger than expected from the sum of the molecular weights of the constituents, suggesting that some components may be present in more than one copy, consistent with reports that the mammalian

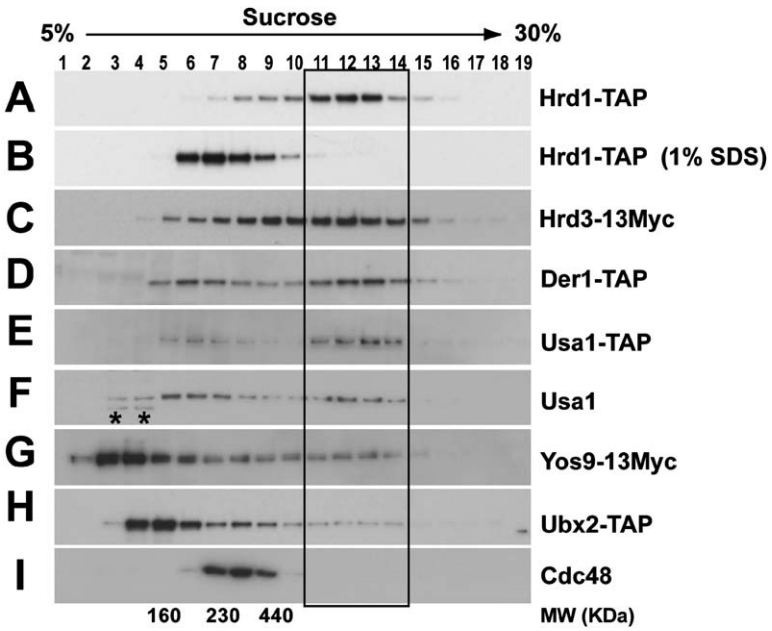

Figure 3. Characterization of the Hrd1p Complex by Sucrose Gradient Centrifugation

Digitonin-solubilized membrane fractions derived from cells expressing various tagged proteins were subjected to centrifugation in a linear 5%–30% sucrose gradient. Fractions were collected and analyzed by immunoblotting with antibodies directed against the tags or the endogenous proteins (in the case of Usa1p and Cdc48p). Stars (*) indicate a nonspecific band recognized by the Usa1p antibodies.

homologs of Der1p form homo-oligomers (Lilley and Ploegh, 2005; Ye et al., 2005).

Usa1p: A Novel ERAD-L Component

Although Usa1p had not previously been described as an ERAD component, it has been found in genome-wide screens to be highly upregulated by the UPR (Travers et al., 2000). Usa1p is predicted to be a double-spanning membrane protein with both N and C termini in the cytosol, and it has an ubiquitin-like (Ubl) domain in its N-terminal cytosolic domain (Figure 4A).

To investigate whether Usa1p has a function in ERAD, we first tested whether the lack of Usa1p compromises the viability of cells that cannot elicit the UPR, as has been observed with other ERAD components (Ng et al., 2000). While cells lacking Ire1p, a key component of the UPR, or cells lacking Usa1p, grew normally at all temperatures tested, a mutant lacking both Ire1p and Usa1p had a severe growth defect at higher temperatures (Figure 4B). Thus, as with other ERAD components, Usa1p becomes essential under stress conditions in cells that cannot elicit the UPR.

Next we compared the degradation kinetics of several ERAD substrates in wild-type and *USA1* deletion cells (Figure 4C). The degradation rates of the ERAD-L substrates KHN, a luminal protein, and of KWW, a membrane protein with a misfolded luminal domain (Vashist and Ng, 2004), were greatly reduced in the absence of Usa1p. The effect was as strong as seen in the absence of Yos9p or Hrd1p. In mutant cells, the molecular weights of the stabilized proteins increased by O-glycosylation (Figure 4C), either in the ER or after export by vesicular trafficking (Vashist et al., 2001). The degradation of the ERAD-L substrate CPY* was also greatly affected in the absence of Usa1p (see below). In contrast, the degradation of the ERAD-C substrate Ste6-166 was not affected by the deletion of *USA1*. Controls showed that, as expected (Huyer et al., 2004), *DOA10* deletion stabilized this substrate (Figure 4D). Thus, in agreement with our observation that Usa1p is associated with known ERAD-L components, Usa1p is functionally required for the ERAD-L but not the ERAD-C pathway.

Yeast Usa1p does not have obvious sequence relatives in higher eukaryotes, but the mammalian Herp protein might perform an analogous function. Like Usa1p, Herp spans the membrane twice and has an N-terminal Ubl domain. It is highly upregulated by UPR, is associated with Hrd1p and Derlin-1, and is required for the degradation of some ERAD substrates (Kokame et al., 2000; Schulze et al., 2005). Consistent with these similarities, we found that the expression of mammalian Herp in a *USA1* deletion strain of *S. cerevisiae* partially restored the degradation of CPY* (Figure 4E) and of KWW (Figure S4).

Hrd1p Is Involved in ERAD-L and ERAD-M Pathways

Although Hrd1p is present in a large core complex (Figure 3), data in the literature suggest that some components may not be required for the degradation of all substrates. For example, while Hrd1p is essential for the degradation of Hmg2p or a mutant version of Pdr5p (Pdr5*), Der1p appears to be dispensable in both cases (see Brodsky and McCracken, 1999). These data suggest that Hrd1p-dependent ERAD substrates fall into different classes. To test this idea, we examined a number of known Hrd1p substrates for their requirement of the Hrd1p-associated proteins. In agreement with the literature, we found that the ERAD-L substrates CPY*, KWW, and KHN, as well as the membrane proteins Hmg2p, Sec61-2, and Pdr5*, which have not yet been classified, are all dependent on Hrd1p for their degradation (Figure 5A and data not shown; for

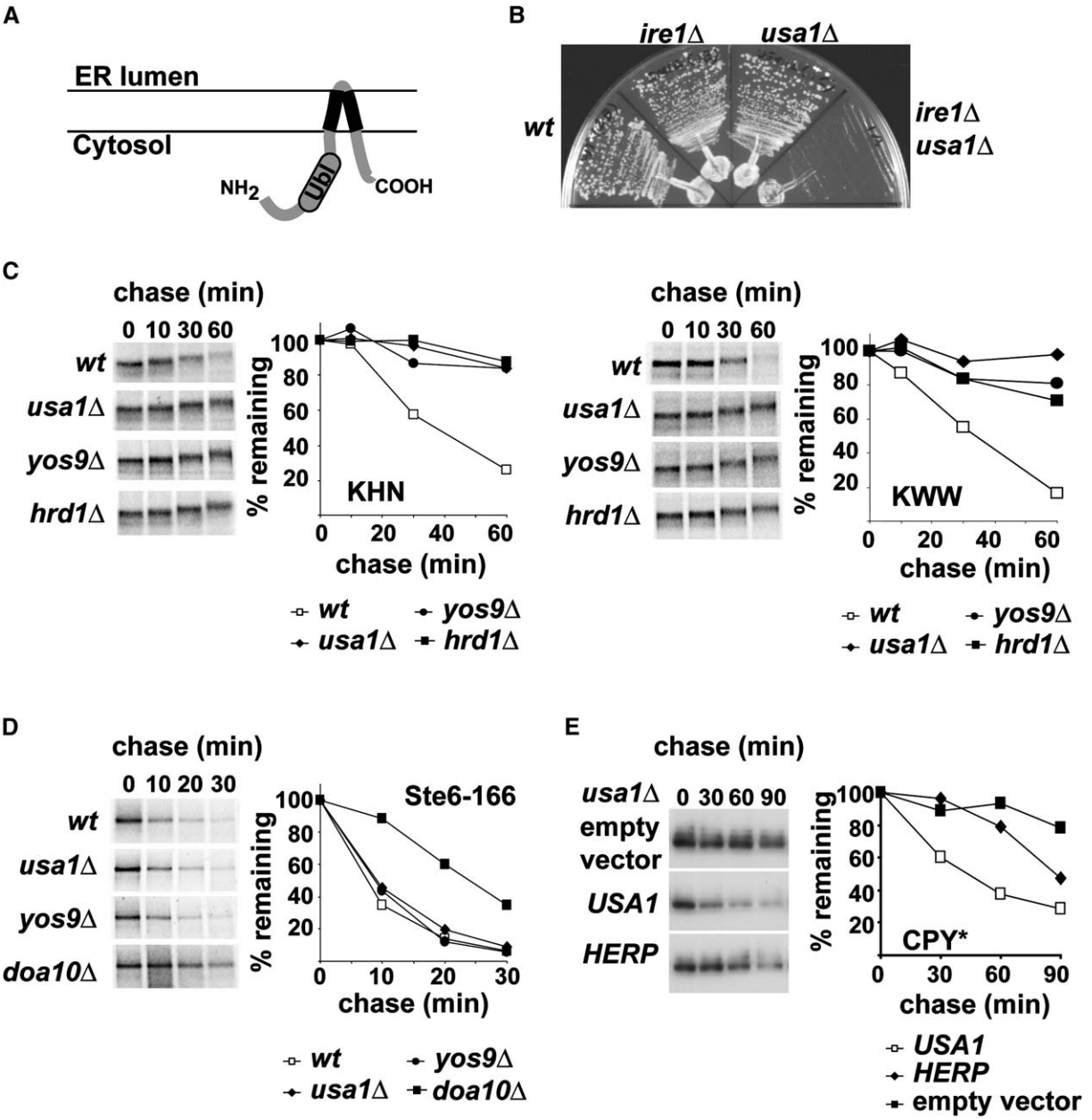

Figure 4. Characterization of the Novel ERAD Component Usa1p

(A) Scheme of the predicted membrane topology and domain structure of Usa1p. Transmembrane segments are shown in black and the Ubl domain is indicated.

(B) Wild-type (*wt*) cells or cells carrying deletions in *USA1* (*usa1Δ*), *IRE1* (*ire1Δ*), or both (*usa1Δ ire1Δ*) were plated on rich medium and incubated at 37°C for 2 days.

(C) To test the degradation of the two ERAD-L substrates KHN and KWW, wild-type (*wt*) cells or cells carrying the indicated deletions were pulse-labeled with ^{35}S-methionine/cysteine and chase-incubated for different time periods. The samples were analyzed by immunoprecipitation, SDS-PAGE, and autoradiography. Densitometry of the bands was used to quantify the data (right panels).

(D) To test the degradation of the ERAD-C substrate Ste6-166, wild-type (*wt*) cells or cells carrying the indicated deletions were analyzed as in (C).

(E) The degradation of the ERAD-L substrate CPY* was tested in *usa1Δ* cells carrying plasmids coding for Usa1p or mammalian Herp. An empty vector was used as a control. Protein degradation was followed by immunoblotting after inhibition of protein synthesis with cycloheximide. The bands were quantitated by densitometry (right panel).

Table 1. Genetic Requirements for the Degradation of Several ERAD Substrates

	ERAD-M		ERAD-L	
Substrates	*HRD1*	*HRD3*	*USA1*	*DER1*
CPY*	+	+	+	+
KHN	+	n.d.	+	+
KWW	+	n.d.	+	+
Sec61-2	+	+	–	–
Hmg2	+	+	–	–
Pdr5*	+	+	–	–
CD4	+	+	n.d.	–

(n.d.) is not determined.

a summary, see Table 1). The absence of Der1p affected CPY*, KHN, and KWW but not Hmg2p, Sec61-2, and Pdr5*, as reported before for some of these proteins. The absence of Usa1p had the same effect as that of Der1p; it affected the ERAD-L substrates CPY*, KHN, and KWW but not Hmg2p, Sec61-2p, and Pdr5* (Figures 4C, 5A, 5B, and S5). Hmg2p, Sec61p-2p, and Pdr5* thus appear to belong to a separate class of ERAD substrates. Hmg2p has a misfolded intramembrane domain in the presence of farnesol (Shearer and Hampton, 2005), Sec61-2p is probably defective inside the membrane since the components of the Sec61p complex do not have significant luminal domains (Van den Berg et al., 2004), and Pdr5* is a multispanning protein with a mutation close to the membrane in a predicted luminal loop. We therefore propose that these ERAD substrates are misfolded inside the membrane and use a pathway, designated ERAD-M, that requires Hrd1p but not Der1p or Usa1p.

Usa1p Provides a Link between Hrd1p and Der1p

Because Hrd1p is involved in both ERAD-L and -M, while the other membrane proteins of the core complex Usa1p and Der1p are only required for ERAD-L, we tested how these components are associated with one another. *DER1* deletion did not affect the interaction between Hrd1p and Usa1p, as determined by sucrose gradient centrifugation (Figures 5C and S6) and by TAP purification

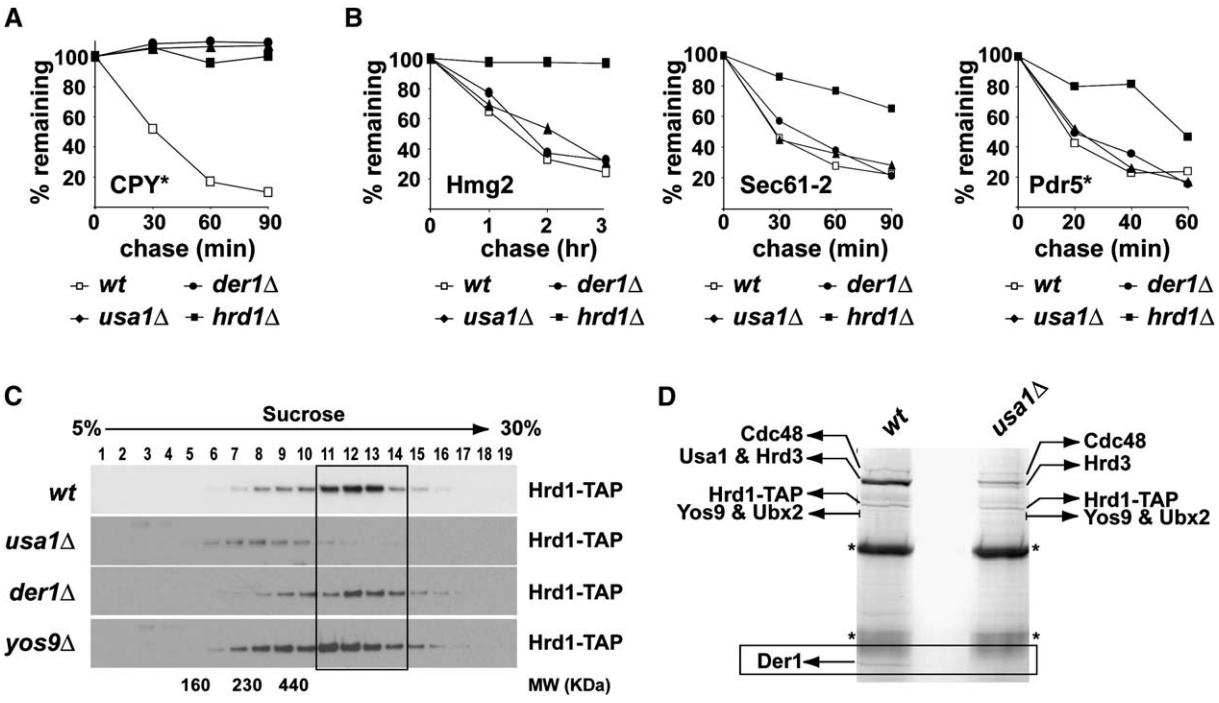

Figure 5. Distinct Hrd1p-Dependent ERAD Pathways

(A) The degradation of the ERAD-L substrate CPY* was analyzed by immunoblotting in wild-type (*wt*) cells or cells carrying deletions of the indicated genes, following inhibition of translation with cycloheximide. The bands of the experiment shown in Figure S5 were quantitated by densitometry.

(B) The degradation of misfolded integral membrane proteins was analyzed as in (A).

(C) Digitonin-solubilized membrane fractions derived from wild-type cells or cells lacking Usa1p, Der1p, or Yos9p were subjected to sucrose gradient centrifugation, and fractions were immunoblotted for Hrd1-TAP as in Figure 3.

(D) Hrd1-TAP was purified on IgG-coupled magnetic beads from wild-type cells or cells lacking Usa1p, and the interaction partners were determined by SDS-PAGE followed by staining with Coomassie blue. The highlighted region was analyzed by mass spectrometry and showed the presence of Der1p only in the sample derived from wild-type cells. Stars indicate the positions of IgG heavy and light chains.

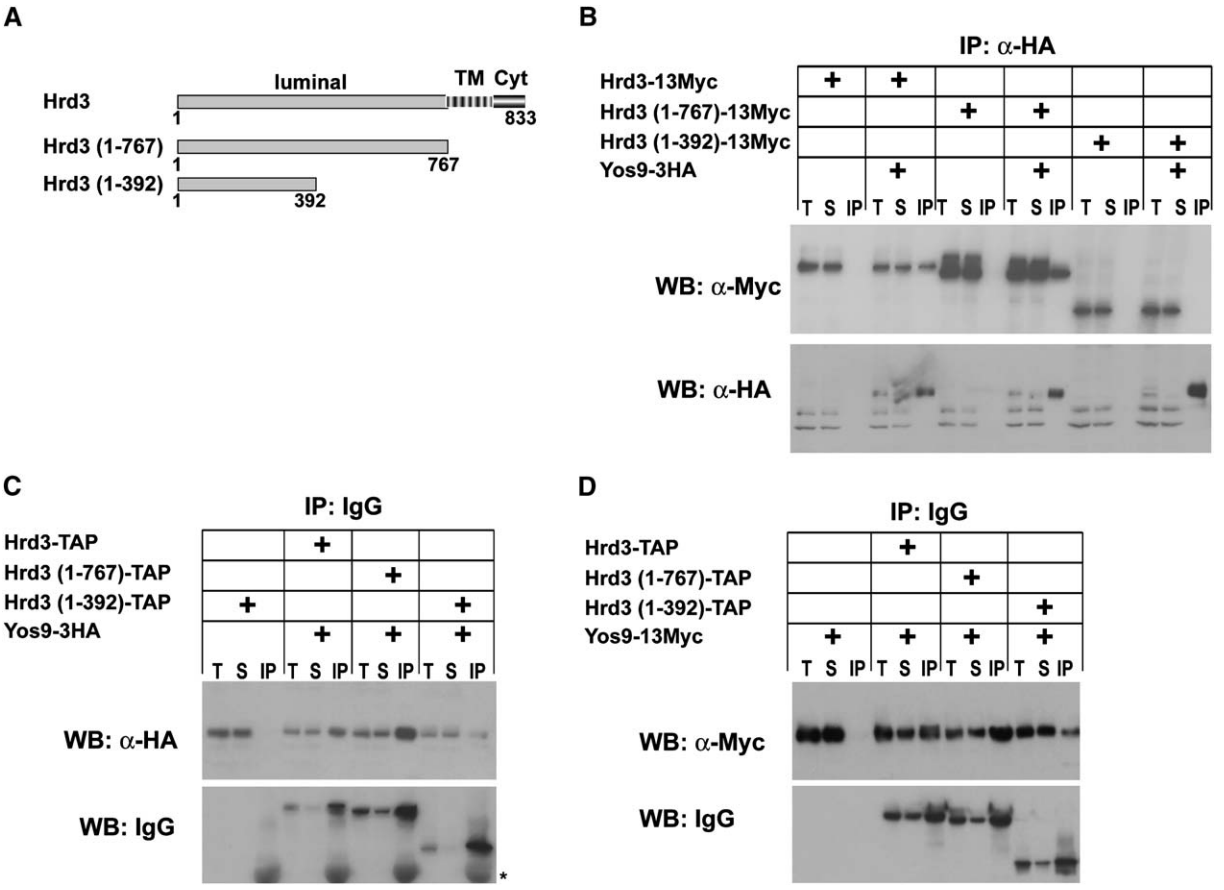

Figure 6. The Luminal Domain of Hrd3p Interacts with Yos9p

(A) Scheme of Hrd3p and of fragments tested for interaction with Yos9p. TM, transmembrane segment; Cyt, cytosolic domain. First and last amino acids are indicated by numbers.

(B) Myc-tagged constructs of full-length Hrd3 or of the fragments shown in (A) were expressed under the endogenous promoter together with HA-tagged Yos9p, also expressed from its endogenous promoter. Cell lysates in digitonin were subjected to immunoprecipitation with HA antibodies and analyzed by SDS-PAGE, followed by immunoblotting with Myc or HA antibodies. T and S represent 7% of the total extract and supernatant, respectively, and IP is the immunoprecipitate. Here, ~16% Yos9p-HA precipitated ~6% of full-length Hrd3p, ~23% Yos9p-HA precipitated ~6% of Hrd3 (1–767), and ~37% Yos9p-HA precipitated <1% of Hrd3 (1–392).

(C) TAP-tagged constructs of Hrd3p were expressed under the endogenous promoter together with HA-tagged Yos9p. Immunoprecipitation was performed with IgG beads, and analysis was done by immunoblotting with HA antibodies or with IgG. T and S represent 10% of the total extract and supernatant, respectively, and IP is the immunoprecipitate. Star (*) indicates the position of IgG heavy chain. Here, ~21% Hrd3p-TAP, ~15% Hrd3 (1–767)-TAP, and ~25% Hrd3 (1–392)-TAP precipitated ~12%, ~14%, and ~4% of Yos9p, respectively.

(D) As in (C), but both the Hrd3p constructs and Yos9-Myc were overexpressed under the Gal promoter for 4 hr. T and S represent 10% of the total extract and supernatant, respectively, and IP is the immunoprecipitate. Here, ~14% Hrd3p-TAP, ~13% Hrd3 (1–767)-TAP, and ~15% Hrd3 (1–392)-TAP precipitated ~9%, ~16%, and ~5% of Yos9p, respectively.

of Hrd1p, followed by mass spectroscopy (data not shown). On the other hand, deletion of *USA1* resulted in a shift of Hrd1p in sucrose gradients to low molecular weight fractions (Figure 5C). Furthermore, when Hrd1p-TAP was purified from cells lacking Usa1p, Der1p was no longer copurified (Figure 5D). These data suggest that Usa1p recruits Der1p to the ubiquitin ligase Hrd1p.

Yos9p Interacts with the Luminal Domain of Hrd3p

Next we wished to analyze how Yos9p, the only luminal protein in the Hrd1p interaction network (Figure 2), is recruited to the ER membrane. We suspected that Hrd3p is the binding partner because it has by far the largest luminal domain among the components of the Hrd1p core complex. Indeed, endogenously expressed Yos9p-3HA pulled down Myc-tagged luminal segments of Hrd3p (Figures 6A and 6B). Conversely, TAP purification of tagged luminal segments of Hrd3p resulted in the copurification of Yos9p-3HA (Figure 6C). With the entire luminal domain of Hrd3p, the interaction was as strong as with full-length Hrd3p, whereas a much weaker interaction was detected with an N-terminal fragment of the luminal domain. The interaction between the luminal domain of Hrd3p and Yos9p is probably direct, because it was observed when both

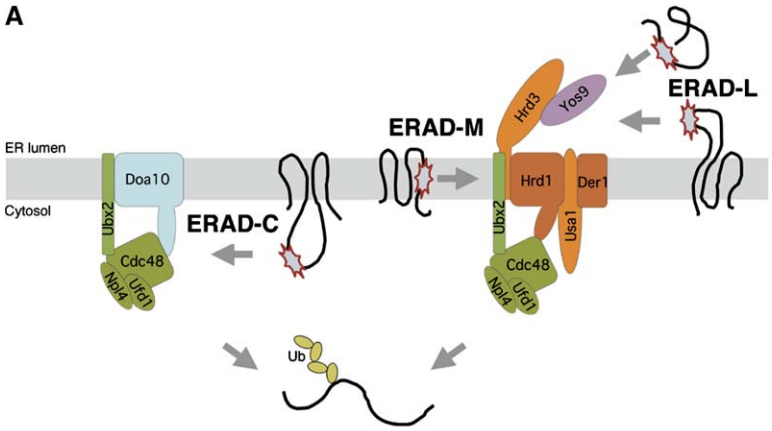

Figure 7. Distinct Ubiquitin-Ligase Complexes Defining Different ERAD Pathways

(A) The scheme shows the ubiquitin-ligase complexes involved in the ERAD-L, -M, and -C pathways. Components in orange and green belong to the Hrd1p core and Cdc48p ATPase complexes, respectively. Stars show the location of the misfolded domain of a substrate. Ub is ubiquitin.

(B) Mammalian homologs or functional equivalents of the components of the yeast ubiquitin-ligase complexes. Question marks (?) indicate uncertainty.

B

S. cerevisiae	*Mammals*
Hrd1	Hrd1; gp78
Hrd3	Sel1
Usa1	Herp
Der1	Derlin-1,-2,-3?
Yos9	Os-9
Doa10	Teb4?
Cdc48	p97
Ubx2	KIAA0887?
Npl4	Npl4
Ufd1	Ufd1

partners were highly overexpressed (Figure 6D). These results suggest that the luminal substrate recognition protein Yos9p delivers its ERAD-L cargoes to the Hrd1 core complex via Hrd3p.

DISCUSSION

We have performed a systematic mapping of interactions between ERAD components in *S. cerevisiae*, which resulted in the identification of most, if not all, components of two distinct ubiquitin-ligase complexes. The complex involving the Hrd1p ubiquitin ligase defines the entire ERAD-L pathway by which proteins with misfolded luminal domains are degraded. Only a subset of the components of the Hrd1 complex is required for the degradation of some membrane protein substrates, and these appear to use a novel pathway, ERAD-M, by which proteins with misfolded intramembrane domains are destroyed. Finally, we identified a complex involving the ubiquitin ligase Doa10p, which defines the ERAD-C pathway that eliminates membrane proteins with misfolded cytosolic domains. The proposed classification is still somewhat preliminary, as it is based on a small number of substrates for each pathway. In addition, proteins might have misfolded domains in more than one location, or the folding of one domain could influence that of another. One ERAD pathway may therefore be dominant over another, as demonstrated for ERAD-C over ERAD-L (Vashist and Ng, 2004), or, in other cases, they might be operating in parallel. It should also be noted that the deletion of most ERAD components does not lead to a complete block of degradation, perhaps because substrates can use an "overflow" pathway involving transport via the Golgi to the vacuole/lysosome (Haynes et al., 2002; Spear and Ng, 2003). Despite these caveats, our results and a large number of data in the literature suggest a simple, unifying concept for ERAD (Figure 7).

The ERAD-L Pathway

As might have been expected, ERAD-L is the most complex pathway, used by substrates with misfolded ER luminal domains, be they in soluble or in membrane proteins. These substrates require a core membrane complex, consisting of the ubiquitin ligase Hrd1p and its cofactors Hrd3p, Der1p, and Usa1p. These components form a near stoichiometric complex that is more loosely associated with the luminal Yos9p and the cytosolic Cdc48 ATPase complex, whose membrane recruitment is facilitated by Ubx2p (Figure 7A).

Yos9p binds misfolded glycoprotein substrates in the ER lumen and may be involved in the initial recognition of at least some ERAD-L substrates (Bhamidipati et al., 2005; Buschhorn et al., 2004; Kim et al., 2005; Szathmary et al., 2005). Yos9p would transfer its cargo to the Hrd1p

core membrane complex, mediated by the demonstrated interaction between Yos9p and Hrd3p. All ERAD-L substrates tested by us are glycoproteins, and it is therefore unclear whether nonglycosylated proteins would employ Yos9p as well. Other luminal factors, such as protein disulfide isomerase or Htm1p, could also play a role in substrate recognition and might deliver their substrates directly to the Hrd1p core complex (Gillece et al., 1999; Jakob et al., 2001). In fact, while cells lacking Hrd3p cannot degrade CPY*, this can be overcome by overexpression of Hrd1p (Plemper et al., 1999), supporting the idea that the Hrd1p core complex can accept substrates from multiple delivery routes, probably by binding to Der1p (Gauss et al., 2006).

In the next step, an ERAD-L substrate must be moved through the membrane, likely through a protein-conducting channel. Possible components of the channel are Der1p and Usa1p because they are only required for substrates with misfolded luminal domains. However, the six transmembrane segments of Hrd1p may also contribute to the formation of a channel. We have not found the Sec61p complex among the interaction partners of ERAD components, and we consider it unlikely that the Sec61p channel is used for ERAD-L substrates because the structure of an archael homolog shows that a "plug" would obscure the access of substrates from the luminal side (Van den Berg et al., 2004).

Because soluble and membrane bound ERAD-L substrates follow the same pathway, we postulate that in the case of a membrane protein, the misfolded luminal domain moves through the channel before the membrane-anchored portion is released into the cytosol. Once a segment of the luminal domain of an ERAD-L substrate emerges into the cytosol, it is polyubiquitylated by the ligase Hrd1p. Our interaction mapping supports the previous conclusion that the ligase cooperates with the ubiquitin-conjugating enzyme Ubc7p and its membrane anchoring protein Cue1p (Biederer et al., 1997; Bordallo et al., 1998). The polyubiquitin chain probably serves both as a ratcheting device, preventing substrate movement back into the ER lumen and as a signal for downstream ubiquitin binding proteins (Flierman et al., 2003).

A critical role of the newly discovered Usa1 protein appears to be as a link between Hrd1p and Der1p in the core complex. Usa1p was found as a prominent interaction partner of all ERAD-L components. It was also detected as a partner of Hrd1p in recent proteomic experiments (Gavin et al., 2006). Usa1p is required for all tested ERAD-L substrates but not for substrates of the other two pathways.

Once polyubiquitylated, a substrate must be moved into the cytosol, likely by the Cdc48p ATPase complex, consisting of Cdc48p itself and the cofactor Ufd1p/Npl4p. Recruitment of the ATPase complex to the membrane probably occurs by several different interactions, with Ubx2p providing a major link through its Ubx domain (Neuber et al., 2005; Schuberth and Buchberger, 2005). Ubx2p spans the membrane twice and has an Uba domain (Schuberth and Buchberger, 2005). How Ubx2p is recruited to the Hrd1p core complex is unknown, but it may involve an interaction with the polyubiquitylated substrate through its Uba domain (Gauss et al., 2006; Neuber et al., 2005), similar to the interaction between the Uba domain in Rad23p and polyubiquitin chains (Richly et al., 2005). The ATPase complex also interacts with substrate-attached polyubiquitin chains (Ye et al., 2003). In addition, from results in mammalian systems it seems possible that Cdc48p interacts directly with the C termini of Der1p and Hrd1p (Ye et al., 2005). Finally, Cdc48p may interact with unfolded segments of the substrate, independently of its polyubiquitin chain (Ye et al., 2003). These multiple interactions would allow the recruitment of the ATPase complex, which in turn would move the substrate into the cytosol for proteasomal degradation.

The ERAD-M Pathway

Data in the literature and our own results indicate that some membrane proteins require for its degradation Hrd1p and Hrd3p, but not the other components of the Hrd1p core complex (Table 1). Although based on a small number of examples, the common feature of these substrates might be a misfolded intramembrane domain. We thus propose that they are degraded by a novel pathway called ERAD-M (Figure 7A). Because Hrd1p and Hrd3p were detected in a single complex, it is possible that the same core complex is employed in both ERAD-M and ERAD-L, although only a subset of the components is functionally required for ERAD-M. Substrates of the ERAD-M pathway may be targeted to the ubiquitin ligase Hrd1p by Yos9p, as is the case for the glycosylated substrate Pdr5*, or by other factors that are too loosely associated to be detected by pull-down experiments. Alternatively, substrates can be targeted directly to Hrd1p, as suggested for Hmg2p by crosslinking experiments (Gardner et al., 2001). The remainder of the pathway would be similar to that of ERAD-L substrates, employing the Cdc48p ATPase complex and the adaptor Ubx2p. Interestingly, ERAD-M substrates do not require the Usa1p and Der1p, two possible channel components. They might either use a smaller channel consisting of Hrd1p alone or directly be extracted from the membrane, as proposed for the bacterial FtsH protein (Akiyama and Ito, 2003). FtsH is an inner membrane protein consisting of two transmembrane segments, an ATPase domain, and a protease domain. It binds to a relatively short cytosolic segment of a misfolded membrane protein, pulls it out of the membrane, and degrades it in a processive fashion. It should be noted that Der1p is required for the degradation of Hrd1p in cells lacking Hrd3p even though Hrd1p does not have a sizable luminal domain (Plemper et al., 1999), but it is unclear whether unstable ERAD components can be considered normal ERAD substrates.

The ERAD-M pathway might be dominant over the ERAD-L pathway. The addition of a transmembrane segment to CPY* converts it into a Der1p-independent ERAD-M substrate (Taxis et al., 2003), and the mammalian

protein CD4 is degraded in *S. cerevisiae* in a Der1p-independent manner even though it has a sizable luminal domain (Meusser and Sommer, 2004). These might be examples where a misfolded luminal domain causes misfolding inside the membrane, or the transmembrane segments might have unusual structures (one segment contains five glycines, the other four tyrosines). In contrast to ERAD-L substrates, the membrane-anchored portion might move into the cytosol before the luminal domain, which would make the ERAD-M pathway dominant.

The ERAD-C Pathway

This pathway takes care of membrane proteins with misfolded cytosolic domains. They appear to be handled in a similar way as ERAD-M substrates, except that they use a different ubiquitin ligase, Doa10p (Figure 7A). Again, substrates may be targeted to the ligase and then moved into the cytosol by the Cdc48p ATPase complex, recruited by Ubx2p. Doa10p can also polyubiquitylate cytosolic and nuclear substrates, so it may be less dedicated to ERAD than Hrd1p (Neuber et al., 2005; Ravid et al., 2006; Swanson et al., 2001). Since proteins with misfolded domains on both sides of the membrane are degraded by the ERAD-C pathway (Vashist and Ng, 2004), we again hypothesize that the membrane-anchored domain is moved into the cytosol before the misfolded luminal domain.

Mammalian ERAD Pathways

We propose that the same three ERAD pathways operate in mammalian cells. As indicated in Figure 7B, there are mammalian homologs of all ERAD components identified in yeast. We show here that the mammalian Herp protein is the functional equivalent to yeast Usa1p even though it does not show significant sequence similarity and is much longer. During evolution, several ERAD components appear to have multiplied and diverged, and they may be required for distinct substrates in mammalian cells. For example, the ligase gp78, but not its cousin Hrd1p, is required for the degradation of mammalian HMG-CoA reductase (Song et al., 2005), and Derlin-2 and -3, but not Derlin-1, are required for the degradation of the NHK protein (Oda et al., 2006). In addition, higher eukaryotes possess additional membrane bound ubiquitin ligases with RING finger domains, and these might have a function in the degradation of some ERAD substrates. Despite this diversification, the mammalian pathways may be fundamentally the same as in yeast. A central role for the Cdc48p ATPase homolog p97 and its cofactor Ufd1/Npl4 is indeed likely. The best characterized mammalian pathway, the viral US11 protein-triggered degradation of MHC class I heavy chains, fits the proposed scheme (Lilley and Ploegh, 2005; Ye et al., 2005): US11 specifically targets its substrate to the ERAD-L pathway, probably replacing the function of cellular Yos9p homologs, by binding to the Derlin-1 protein and a ubiquitin ligase. Because US2-dependent degradation appears to be independent of Derlin-1, it seems possible that it sends MHC class I heavy chains to the ERAD-M pathway. In mammals, but not in yeast, the targeting of HMG-CoA reductase to the gp78 ligase and p97 ATPase complex appears to be mediated by INSIG, which binds the substrate in a sterol-dependent manner (Flury et al., 2005; Song et al., 2005). These results indicate that in mammals there are additional pathways by which ERAD substrates are recognized, but the core machinery may be conserved among all eukaryotes.

EXPERIMENTAL PROCEDURES

Yeast Strains and Plasmids

Tagging of proteins and gene deletions were performed using standard PCR-based homologous recombination. The strains used are isogenic to BY4730 (*Mata ura3D0 his3D1 leu2D0 met15D0*) and are given in Table S7. Details on the plasmids coding for Usa1p (pPC254) and FLAG-Herp (pPC290) are available in the Supplemental Data section.

Protein Purification

The membranes of ~15 g of cells were sedimented and solubilized in lysis buffer containing 1% digitonin. The extract was incubated with either IgG agarose beads (Pharmacia) or with IgG-coupled magnetic beads (Dynal) for 3–4 hr. After washing, the bound proteins were eluted with either TEV protease (Rigaut et al., 1999) or by SDS, respectively. The TEV-eluted proteins were bound to a calmodulin column (Pharmacia) and, after washing, elution was performed with EGTA. Eluted proteins were analyzed by SDS-PAGE, and individual bands were excised and subjected to mass spectrometry. Alternatively, the total population of eluted proteins was precipitated with trichloroacetic acid and analyzed by mass spectrometry. Details of the purification are given in the Supplemental Data section.

Antibodies to Usa1p

Polyclonal antibodies were raised by Zymed in rabbits against a carrier-conjugated peptide corresponding to the C terminus of Usa1p (positions 820–838).

Immunoprecipitation

Crude membrane fractions were prepared from 100 ml cultures. The membranes were solubilized in digitonin, as for TAP purification, and proteins were subjected to immunoprecipitation with either HA antibodies or IgG, both covalently coupled to beads. After washing, the bound proteins were eluted with SDS, separated by SDS-PAGE, and analyzed by immunoblotting with antibodies to HA or Myc, or with IgG.

Sucrose Gradient Centrifugation

Crude membrane fractions were prepared from 100 ml cultures. The membranes were solubilized in digitonin, as for TAP purifications, and the extract (0.3 ml) was applied to a 5%–30% linear sucrose gradient, generated with a Biocomp gradient maker. Centrifugation was performed at 50 K in a SW55 rotor (Beckman) for 4 hr at 25°C. Fractions (~0.25 ml) were collected from the top and analyzed by SDS-PAGE and immunoblotting.

ERAD-Substrate Degradation Experiments

Plasmids coding for the ERAD substrates were generous gifts from R. Hampton, S. Jentsch, S. Michaelis, D. Ng, and D. Wolf. Cycloheximide shut-off experiments were performed in exponentially growing cells, as described (Gardner et al., 2000). Pulse-chase experiments were performed in exponentially growing cells, as described (Vashist and Ng, 2004).

Supplemental Data

Supplemental Data include six figures, one table, and some experimental procedures and can be found with this article online at http://www.cell.com/cgi/content/full/126/2/361/DC1/.

ACKNOWLEDGMENTS

We would like to thank R. Hampton, S. Jentsch, S. Michaelis, D. Ng, D. Pellman, R. Schekman, J. Weissman, D. Wolf, and Y. Ye for yeast strains and reagents; A. Rudner for suggesting the use of magnetic beads; R. Tomaino for technical advice; D. Finley and Y. Ye for critical reading of the manuscript; and members of the Rapoport lab for discussions. P.C. was supported by a Jane Coffin Childs Memorial Fund for Medical Research fellowship. T.A.R. is supported by NIH grant GM052586 and is a Howard Hughes Medical Institute Investigator.

Received: February 17, 2006
Revised: April 30, 2006
Accepted: May 18, 2006
Published: July 27, 2006

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